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TITLE PAGE

CYTISINE-BASED NICOTINIC PARTIAL AGONISTS AS NOVEL ANTIDEPRESSANT COMPOUNDS

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Nonstandard abbreviations:

3-pyr-Cyt: 3-(pyridin-3'-yl)-cytisine

5-Br-Cyt: 5-bromo-cytisine

ABSTRACT

Nicotine and other nicotinic agents are thought to regulate mood in human subjects and have antidepressant-like properties in animal models. Recent studies have demonstrated that blockade of nicotinic acetylcholine receptors (nAChRs) including those containing the β2 subunit (β2*), results in antidepressant-like effects. Previous studies have shown that cytisine, a partial agonist at α4/β2* nAChRs, and a full agonist at α3/β4* and α7 nAChRs, has antidepressant-like properties in several rodent models of antidepressant efficacy; however, it is not clear whether more selective partial agonists will also be effective in these models. We tested cytisine and two derivatives, 5-bromo-cytisine (5-Br-Cyt) and 3-(pyridin-3'-yl)-cytisine (3-pyr-Cyt) for their ability to act as a partial agonist of different nAChR subtypes and to show antidepressant-like activity in C57/BL6 mice in the tail suspension, the forced-swim, and the novelty-suppressed feeding tests. 3-pyr-Cyt was a partial agonist with very low efficacy at α4/β2* nAChRS but had no agonist effects at other nAChRs normally targeted by cytisine, and was effective in mouse models of antidepressant-efficacy. Animals showed dose-dependent antidepressant-like effects in all three behavioral paradigms. 5-bromo-cytisine (5-Br-Cyt) was not effective in behavioral tests when administered peripherally, likely due to its inability to penetrate the blood-brain barrier, since it efficiently reduced immobility in the tail suspension test when administered intraventricularly. These results suggest that novel nicotinic partial agonists may provide new possibilities for development of drugs to treat mood disorders.

INTRODUCTION

Numerous studies have suggested that tobacco smoke can modulate depressive symptoms in human subjects and that nicotinic agents can have antidepressant-like effects in animal models (Picciotto et al., 2002). Individuals with a history of depression have an approximately 50% higher incidence of smoking than the general population (Glassman et al., 1990). In addition, nicotine ((S)-3-(1-Methyl-2-pyrrolidinyl)pyridine) patch can reduce symptoms of depression in non-smokers (Salin-Pascual et al., 1995) whereas smoking cessation can exacerbate symptoms of depression (Glassman et al., 1990). Animal studies have also shown that chronic nicotine administration can elicit antidepressant-like effects in rats both in the learned helplessness (Semba et al., 1998) and the forced swim (Djuric et al., 1999; Tizabi et al., 1999) paradigms.

Nicotine exerts its effects by binding to, activating and desensitizing nicotinic acetylcholine receptors (nAChRs) in the central nervous system and autonomic ganglia (Picciotto et al., 2008). $\alpha4/\beta2$* receptors (where * indicates the possible inclusion of other nAChR subunits, such as $\alpha5$, $\alpha6$ or $\beta3$ along with $\alpha4$ and $\beta2$) are the most widely expressed nAChRs in the central nervous system, and also have the highest affinity for nicotine, whereas $\alpha7$* nAChRs, which are also found at high levels in brain, form functional homomers and are highly expressed in the hippocampus and cortex, but also found in most other brain regions (Zoli et al., 1998). In the peripheral nervous system, $\alpha3/\beta4$* nAChRs are the main ganglionic subtype, but are only expressed at relatively low levels in the brain (Gotti et al., 2006). Compared to acetylcholine (2-acetoxy-N,N,N-trimethylethanaminium), the endogenous ligand of nAChRs, nicotine acts much like a partial agonist (Papke et al., 2007).

It appears somewhat paradoxical that increased endogenous acetylcholine levels results in depression-like symptoms (Janowsky et al., 1972) whereas nicotine administration can decrease

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depressive symptoms (Salin-Pascual et al., 1995); however, chronic nicotine administration can desensitize nAChRs (Reitstetter et al., 1999), resulting in functional antagonism (for reviews see (Quick and Lester, 2002; Picciotto et al., 2008). Thus, blockade (i.e. antagonism and/or receptor desensitization) rather than activation of nAChRs might have antidepressant effects in human subjects. This hypothesis is supported by the fact that mecamylamine ((1R,2R,4S)-N,2,3,3-tetramethylbicyclo[2.2.1]heptan-2-amine), a non-selective nAChR antagonist, decreases symptoms of depression in patients with Tourette's syndrome (Janowsky et al., 1972) and has antidepressant-like effects in mice (Caldarone et al., 2004; Rabenstein et al., 2006; Mineur et al., 2007c). Similar antidepressant-like effects have been observed in mice treated with cytisine ((1R,5S)- 1,2,3,4,5,6- hexahydro- 1,5-methano- 8H-pyrido [1,2a] [1,5] diazocin- 8-one) (Mineur et al., 2007c), a quinolizidine alkaloid isolated from the seeds of *Cytisus sp.* and related members of *Leguminosae* (*Fabaceae*). Cytisine is a partial agonist at β2* nAChRs and a full agonist at β4* nAChRs (Papke and Heinemann, 1994; Picciotto et al., 1995). Taken together, these data indicate that blockade of β2* nAChRs may be critical for the antidepressant-like effects of nicotinic agents. Similarly, studies in knockout mice have demonstrated that the absence of β2* nAChRs throughout development can lead to antidepressant-like phenotypes (Caldarone et al., 2004) and these mice are insensitive to the antidepressant-like effects of the nicotinic antagonist mecamylamine (Rabenstein et al., 2006). Moreover, amitriptyline , a tricyclic antidepressant, has no effect in β2 knockout mice (Caldarone et al., 2004) strongly suggesting that β2* nAChRs are (3-(10,11-dihydro-5H-dibenzo[[a,d]] cycloheptene-5-ylidene)-N, N-dimethyl-1-propanamine) important for the function of classical antidepressants. Indeed, it has been proposed that the antidepressant effects of fluoxetine (N-methyl-3-phenyl-3-[4- (trifluoromethyl)phenoxy]propan-

1-amine) could involve the acceleration of nAChR desensitization (Garcia-Colunga & Miledi, 1997).

Mecamylamine has been successfully used to treat depression in combination with selective serotonin reuptake inhibitors (SSRIs) in clinical trials to treat patients resistant to the SSRIs alone (George et al., 2008); however, mecamylamine can result in side effects including dysregulation of the autonomic system, likely due to its ability to block ganglionic α3/β4* nAChRs. Similarly, cytisine can decrease the effects of endogenous ACh at β2* nAChRs due to its high affinity and low efficacy as a partial agonist, but cytisine is a full agonist at α3/β4* nAChRs at low concentrations, which results in autonomic activation and potential toxic side effects (Etter, 2006). Based on these observations, we started to investigate cytisine derivatives to identify compounds with greater selectivity for β2* nAChRs but relatively low affinity and efficacy at α3/β4 nAChRs (Fitch et al., 2005). We hypothesized that compounds that were more selective partial agonists of β2* nAChRs would have antidepressant-like effects in rodent behavioral models, with higher efficacy (and potentially fewer side effects).

METHODS

Synthesis

Compounds were derived as previously described (Imming et al., 2001; Fitch et al., 2005). Briefly, cytisine was obtained by isolation from seeds and pods from *Laburnum anagyroides* and *Laburnum watereri* and was subsequently protected at the secondary nitrogen with N-*t*Boc-anhydride. Halogenation was performed with N-bromosuccinimide for introduction of a bromo substituent on the N-*t*Boc-cytisine. The resulting products were 3- and 5-bromo

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derivatives along with the 3,5-dibromo derivative, which were subsequently separated by column chromatography on silica.

3-(Pyridin-3'-yl)-cytisine: The 3-bromo derivative was further derivatized by introducing the pyridine-3-yl moiety from the corresponding pyridine-3-boronic acid via a Suzuki cross-coupling reaction under inert atmosphere in a mixture of 1,2-dimethoxyethane/water and $Pd(PPh_3)_4$ as a catalyst by means of microwave irradiation. The crude reaction mixture was liberated from the catalyst by extraction on a SPE C-18 column with a methanol water mixture and was purified by HPLC on a reversed phase column with a methanol/water gradient.

Both N-*t*Boc-protected derivatives were cleaved from the *t*Boc protection group by means of microwave irradiation in pure water. Subsequent lyophilization yielded the compounds at > 99 % purity (LCMS). 5-bromo-cytisine was selected because it showed an antagonist-like profile for α4/β2 nAChRs. In contrast, 3-bromo cytisine is an agonist. We therefore investigated whether a 3-substituted compound with a larger substituent might bias the functional properties towards partial agonism.

Cytisine and its derivatives were tested by radioligand binding assays for their abilities to compete for [^{3}H]epibatidine and [^{3}H]MLA binding sites in rat forebrain (α4/β2*, α7*), pig adrenals (α3/β4*) and *Torpedo californica* electroplax ($(\alpha1)_2\beta1\gamma\delta$) membrane fractions as has been described (Gundisch et al., 1999; Mukhin et al., 2000; Gohlke et al., 2002; Fig. 1).

ACh receptor clones

Human nAChR receptor clones and α4/β2 concatamers were the generous gift of Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia PA).

Expression in Xenopus laevis oocytes

Mature (>9 cm) female *Xenopus laevis* African frogs (Nasco, Ft. Atkinson, WI) were used as a source of oocytes. Before surgery the frogs were anesthetized by placing them in a 1.5 g/l solution of MS222 for 30 min. Oocytes were removed from an incision made in the abdomen.

Harvested oocytes were treated with 1.25 mg/ml collagenase (Worthington Biochemical Corporation, Freehold NJ) for two hours at room temperature in calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 2.38 mM $NaHCO_3$, 0.82 mM $MgS0_4$, 15 mM HEPES (pH 7.6), 12 mg/l tetracycline) in order to remove the follicular layer. Stage-5 oocytes were isolated and injected with 50 nl (5-20 ng) of each subunit cRNA (Papke and Heinemann, 1994; Papke and Porter Papke, 2002; Papke et al., 2007). Recordings were normally conducted 2-5 days post-injection.

Electrophysiology

Experiments were conducted using OpusXpress6000A (Molecular Devices, Union City, CA). OpusXpress is an integrated system that provides automated impalement and voltage clamp of up to eight oocytes in parallel. Both the voltage and current electrodes were filled with 3M KCl. The oocytes were clamped at a holding potential of -60 mV.

Data were collected at 50 Hz and filtered at 20 Hz for $\alpha7$ and at 5 Hz for $\alpha4\beta2$. The oocytes were bath-perfused with Ringer's solution. Agonist solutions were delivered from a 96-well plate using disposable tips. Flow rates were set at 2 ml /min for $\alpha7$ and 4 ml/min for $\alpha4/\beta2$.

Experimental protocols and data analysis

Responses of α7 nAChRs were calculated as net charge (Papke and Porter Papke, 2002), and responses of other nAChR subtypes are reported as peak currents. Each oocyte received initial control applications of ACh, then experimental drug applications, and follow-up control applications of ACh. The optimal control ACh concentrations were empirically determined in separate experiments. For α7 nAChRs, the control ACh concentration was 300 μM, for α3β4 nAChRs the ACh control was 100 μM and for α4β2 nAChRs formed following the injection of α4 and β2 RNA at 1:1 ratio the ACh control was 30 μM. Since the injection of RNAs at the 1:1 ratio typically produces a heterogeneous population of receptors, with two alternative subunit stoichiometries (α4(2)β2(3) and α4(3)β2(2)), we used a concatamer of the α4 and β2 subunits (Zhou et al., 2003) to force the formation either α4(2)β2(3) receptors which have a high sensitivity to relatively low concentrations of ACh or nicotine (HS α4β2) or α4(3)β2(2) receptors which generate larger current but require relative high concentrations of ACh or nicotine for maximal activation ACh (LS α4β2). Specifically the β2-6-α4 concatamer was coexpressed with either monomeric α4 or β2 to generate either the low sensitivity (LS) or high sensitivity (HS) forms of the receptors, respectively. In these receptors, the agonist binding sites are within each of the two concatamers and the monomeric subunit forms the fifth structural subunit in the pentamer, although this has not been established for cytisine and its analogues. For the high and low sensitivity α4/β2 nAChRs formed with concatamers, the ACh controls were 10 μM and 100 μM, respectively. Responses to experimental drug applications were calculated relative to the preceding ACh control responses in order to normalize the data, compensating for the varying levels of channel expression among the oocytes. Mean values and standard errors (SEM) were calculated from the normalized responses of at least four oocytes for each experimental concentration. Values measured relative to the ACh controls were expressed

relative to ACh-evoked maximal responses based on separate ACh concentration response studies (not shown) that provided the ratio between the ACh control responses and the maximal ACh-evoked responses for each receptor subtype. For concentration-response relationships, data were plotted using Kaleidagraph 3.0.2 (Abelbeck Software; Reading, PA), and curves were generated from the Hill equation:

$$\text{Response} = \frac{I_{Max}[\text{agonist}]^n}{[\text{agonist}]^n + (EC_{50})^n}$$

where I_{max} denotes the maximal response for a particular agonist/subunit combination, and n represents the Hill coefficient. I_{max}, n, and the EC_{50} were all unconstrained for the fitting procedures.

Animals

Three-month-old C57BL/6J (B6) male mice (25 to 30 grams) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were group-housed (5 per cage) under standard laboratory conditions (temp. 21±2^{o}C, 12:12 light-dark cycle, lights on at 7:00 a.m.) with food and water available *ad libitum,* unless otherwise noted. At least two weeks of rest followed their arrival; during that time, mice were marked on their tail with a permanent marker for identification and were randomly assigned to one of the different treatment groups (n=10 per group unless otherwise stated). All procedures were approved by the Yale University Animal Care and Use Committee.

General testing conditions

Mice were habituated to the testing room at least 30 min before any behavioral evaluation. All tests took place between 12:00 noon and 5 p.m. The testing area was dimmed to limit stress or anxiety. During behavioral evaluation, the experimenters were blind to treatment.

Treatments - Experimental design

The concentrations of the compounds used in this study were based on previous studies using cytisine (Mineur et al., 2007c), on the relative affinities of each compound for α4/β2 nAChRs, their molecular weight, and their availability. Pilot studies also helped refine the dose range used in behavioral assays. Additionally, we found that using higher doses than those described here were not well tolerated by the mice and led to decreased locomotion, confounding the outcome of behavioral testing. Cytisine (0.3, 0.6, 1. and 1.5 mg/kg), 5-Br-Cyt (0.3, 0.6, 0.9, 1.2 mg/kg), 3-pyr-Cyt (0.3, 0.6, 0.9 mg/kg), nicotine (expressed as free-base: 0.03, 0.09, 0.5, 1 mg/kg), and fluoxetine (used as a positive control: 10 mg/kg) were diluted in phosphate buffered saline (Saline). Saline or drugs were injected *i.p.* 30 min prior to the tail suspension test or the forced swim test. The two tests were separated by 48 hr. After the latter test, saline or drug was injected daily (between 9 and 11 a.m.) for 15 days and mice were subsequently tested in the novelty suppressed feeding test. Testing for novelty suppressed feeding was performed before daily injection to avoid acute effect of the drugs. Nicotine was not used in this test because it decreases appetite, confounding the motivation to seek food in the novelty suppressed feeding test (Mineur et al, 2007)

For the experiment with intraventricular infusion (i.c.v.) of 5-Br-Cyt, 20 naïve mice (10 5-Br-Cyt-treated and 10 vehicle-treated) underwent canulation surgery. A concentration of 50 ng

5-Br-Cyt/1 µl/animal was used based on the molarity of the compound, its pharmacological properties and comparisons to the effects of the other compounds. The solution was prepared in artificial cerebrospinal fluid (aCSF) and was infused slowly over ~30 seconds, 20 to 30 minutes before the beginning of the tail suspension test. The animals from the control group (aCSF) received only artificial cerebrospinal fluid.

Surgery

Mice were anesthetized with a mixture of xylazine/ketamine diluted in saline (100 mg/kg and 10 mg/kg respectively) at 10 ml/kg. After complete anesthesia, mice were placed in a stereotaxic frame, and one guide canula (3mm pedestal; designed to receive canulas of gauge 33; Plastic One, Roanoke, VA) was implanted per animal (from Bregma: Antero-Posterior: +1.1mm; Lateral: 1 mm; Ventral: -3.5 mm). Following canulation, mice were singly housed and allowed to recover for one week before testing.

After testing, mice were irreversibly anesthetized with chloral hydrate and canula placement was checked. After verification of placement, 7 control- and 8 5-Br-Cyt-treated animals were used for subsequent analysis.

Behavioral assays

Mice underwent three behavioral tests sequentially: the tail suspension test, followed by the forced-swim 48 hours later, followed by the novelty-suppressed feeding 15 days later. While somewhat stressful, the tail suspension and forced swim tests are of short duration and 48 hours of rest was observed between each paradigm. Mice were not handled before testing. The novelty suppressed feeding test took place 15 days after the tests sensitive to acute antidepressant

treatment. C57BL/6J males are particularly resistant to stress, even chronic stressors (see Mineur et al., 2006; Mineur et al., 2007a). Previously published articles have used a similar design and we have validated the performance in single vs. repeated tests in C57BL/6 mice with several antidepressant-like compounds and mecamylamine (Rabenstein et al., 2006; Mineur et al., 2007b; Mineur et al., 2007c). This design allows us to reduce the number of animals used for these studies resulting in an "n" of 10 for each dose. Each mouse is subjected to the same tests, from less stressful to more stressful as has been recommended (Crawley, 2008). In previous experiments, we have never seen a difference in animals subjected to the forced swim test alone or after the tail suspension test (data not shown). Similarly, the novelty-suppressed feeding test is not affected by earlier testing in the forced swim test in our hands (data not shown).

Tail suspension test

Mice were suspended by the tail by gently taping the tail to a paper clip that was then attached to a length of string, and time spent immobile was recorded over a 6 min period (Mineur et al., 2006). After completion of the test, mice were returned to a holding cage until all cage-mates were tested. At the end of all experiments, mice were returned to their home cage and transferred back to the holding room.

Forced swim test

Mice were gently placed in a 4-liter beaker (18 cm in diameter) filled with 15 cm of water (~ 25-26 ºC) to prevent mice from touching the bottom of the beaker with their paws or tail. Time spent immobile during the 15-min testing period was recorded. The 15 min test was chosen based on our prior experience with C57BL/6J mice, since this strain shows very little

immobility during the first 5 min of testing (Caldarone et al., 2003; Caldarone et al., 2004; Mineur et al., 2006), the time point often used in other strains of mice and in rats. After testing, each mouse was placed in a warm holding cage (30-35^{o}C) with bedding covered by a paper towel. After each mouse was tested, animals were returned to the holding room.

Acute locomotor activity measurements

Mice were placed in a clean Plexiglas cage (48 x 22 x 18 cm) for 20 min after injection of one of the nicotinic compounds. Locomotor activity was recorded using the Optomax system (Columbus instruments, Columbus, Ohio, USA). All mice from the same cage were tested at the same time in separate locomotor boxes. Subjects were returned to their home cage at the end of the test.

Novelty-suppressed feeding test

The protocol for novelty-suppressed feeding was based on previously published paradigms (for a review, see (Dulawa et al., 2005). After 15 days of drug treatment, mice were weighed and food was removed from the cage. Twenty four hours later, mice were transferred to the testing room, weighed again, placed in a clean holding cage and allowed to habituate for at least 30 min. The testing apparatus consisted of a clear Plexiglas enclosure (40 x 40 x 17 cm), with a lid. The floor was covered with 2 cm of corncob bedding. A small piece of mouse chow was placed in the center of the arena on a piece of white circular filter paper (diameter: 9.5 cm). At the start of the experiment, each mouse was placed in the corner of the testing area, and the time to the first feeding event was recorded. Immediately after the mouse began to eat, the subject was placed alone for 5 min in its original home cage with a pre-weighed piece of lab

chow. At the end of the 5-min period, the amount of food consumed was determined. After all mice from a single cage were tested, mice were returned to their home cage.

Statistical analyses

Data from the behavioral assays were evaluated with analyses of variance (ANOVA) with "treatment" and "concentration" as between subject factors. When relevant, posthoc analyses were performed by t-tests with Bonneferonni/Dunnet's correction for multiple comparisons. Alpha was set at 5%.

RESULTS

Electrophysiologial properties of 3-pyr-Cyt and 5-Br-Cyt

The electrophysiologial properties of 3-pyr-Cyt and 5-Br-Cyt are summarized in Table 1. Cytisine (Fig. 1A) was less potent but more efficacious at low sensitivity (LS) α4β2 receptors (concatamers containing 3 α4 and 2 β2 subunits; I_{max} 10% that of ACh; Fig. 1B) compared to high sensitivity (HS) α4β2 receptors (concatamers containing 2 α4 and 3 β2 subunits). In contrast, 3-pyr-Cyt (Fig. 1C) is a relatively weak partial agonist for both the LS and HS receptors (efficacy 8% and 3%, respectively) with similar potencies for both forms of α4β2 nAChRs (Fig. 1D). Another cytisine derivative, 5-Br-Cyt (Fig. 1E) showed no significant differences in its activity at LS and HS forms of α4/β2 nAChRs (Fig. 1F) and was a partial agonist with approximately 16% the efficacy of ACh.

Consistent with what has been reported for the effects of cytisine (Sigma; Papke et al., 2007) on rat nAChRs expressed in oocytes , 5-Br-Cyt was relatively efficacious at α7 and α3β4 nAChRs (Fig. 2A). In contrast, 3-pyr-Cyt (Fig. 2B) produced very little activation (≤ 5% ACh

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maximum) of α3β4 or α7 receptors at concentrations ≤ 100 μM, making it a weak partial agonist for all of these neuronal nAChR subtypes. The partial agonist activity of 3-pyr-Cyt for α4β2 nAChRs was further studied in ACh co-application experiments. As expected, co-application of the partial agonist 3-pyr-Cyt with the full agonist ACh to cells expressing α4β2 nAChRs, resulted in decreased responses compared to those produced by ACh alone, dependent on 3-pyr-Cyt concentration and limited by the intrinsic activity of 3-pyr-Cyt (Fig. 2C). There was no evidence for residual desensitization produced by the compounds used in the present study (not shown). That is, ACh controls recorded 5 minutes after the application of the experimental compounds were not significantly different from those recorded prior to experimental drug application, throughout the concentration ranges tested. While this experiment confirms that 3-pyr-Cyt can bind to α4β2 receptors and limit ACh responses, it should be noted that this co-application protocol certainly underestimates the potency that 3-pyr-Cyt would have for similar effects *in vivo.* In a therapeutic context, the potency of 3-pyr-Cyt for modulating endogenous cholinergic signals following systemic delivery would most likely correspond to its very high affinity for inducing and maintaining α4β2 receptors in the desensitized state, as measured by binding studies.

Behavioral effects of nicotine, cytisine, 3-pyr-Cyt and 5-Br-Cyt

Tail suspension test

There was a dose-dependent effect of 3-pyr-Cyt and cytisine, but not 5-Br-Cyt on immobility in the tail suspension test ($F(3, 48) = 3.18$, $p = 0.032$, $F(3, 47) = 4.46$, $p = 0.007$ and $F(4, 44) = 0.58$, $p = 0.68$, respectively (Fig. 3A-**C**).

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Post hoc t-test analyses revealed that mice injected with 3-pyr-Cyt spent significantly less time immobile at 0.6 mg/kg ($p = 0.012$) while the effects of cytisine only reached significance at 1 mg/kg ($p = 0.004$). At a higher dose, cytisine (1.5 mg/kg) still showed significant effects in the tail suspension test ($p = 0.016$) whereas higher doses of 3-pyr-Cyt (0.9 mg/kg) showed a trend that did not reach significance ($p = 0.14$). Comparatively, there was a significant main effect of nicotine ($F(4, 55) = 4.86$, $p = 0.002$); however post hoc analyses revealed that this interaction was mainly due to a significant difference between 0.03 mg/kg and 1 mg/kg ($p < 0.0001$) while no significance was found between saline treatment and any of the doses of nicotine used in this study (Fig. 3D). Fluoxetine induced a significant reduction of immobility at 10 mg/kg compared to saline ($F(1, 19) = 6.05$, $p = 0.026$ (Fig. 3D).

Forced-swim test

Mice treated with cytisine or 3-pyr-Cyt, but not 5-Br-Cyt, showed a dose-dependent decrease in immobility in the forced swim test ($F(3, 44) = 11.58$, $p < 0.0001$, $F(3, 40) = 10.51$, $p < 0.0001$ and $F(4, 40) = 1.80$, $p = 0.14$, respectively (Fig. 4A-C). Post hoc t-tests indicated that mice were significantly less immobile when they were treated with 0.3, 0.6 or 0.9 mg/kg 3-pyr-Cyt ($p = 0.015$, $p < 0.0001$ and $p = 0.0014$, respectively). Mice treated with cytisine were significantly less immobile at doses of 0.75 mg/kg and 1 mg/kg ($p = 0.014$ and $p < 0.0001$, respectively), but this effect was not seen at 1.5 mg/kg. Acute nicotine treatment had no significant main effect in the forced swim test ($F(4, 55) = 1.17$, $p = 0.33$; Fig 4D); however, fluoxetine resulted in a significant reduction of immobility at 10 mg/kg compared to saline treated animals ($F(1, 16) = 5.34$, $p = 0.034$ (Fig. 4D).

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Locomotor activity

No overall treatment effect of any of the compounds tested was detected on acute locomotor activity ($F(4, 45) = 1.58$, $p = 0.33$; Fig 5).

Novelty-suppressed feeding

As has been reported previously (Mineur et al., 2007c), chronic (15 days), but not acute, cytisine treatment decreased the latency to initiate feeding in the novelty-suppressed feeding test as compared to saline treatment (Fig. 6A; $F(2, 32) = 2.42$, $p = 0.01$) but post hoc t-test analyses show that this effects only reached significance at a dose of 1 mg/kg ($p = 0.0001$). A significant effect of 3-pyr-Cyt was also observed (Fig 6B, $F(2, 33)=3.39$, $p = 0.04$), and post hoc t-test analyses revealed that this effect was only significant at 0.3 mg/kg 3-pyr-Cyt ($p = 0.023$) but not at 0.6 mg/kg ($p = 0.52$). No significant effect of 5-Br-Cyt was seen in the novelty-suppressed feeding test (Fig. 6C, $F(3, 36) = 0.10$, $p = 0.95$). Comparatively, fluoxetine induced a significant decrease in the time to first feed at 10 mg/kg compared to saline ($F(1, 13) = 9.09$, $p = 0.009$; Fig 6D). No effects on homecage food consumption (5 min in home cage) or body weight were observed at any doses of cytisine, 3-pyr-Cyt, 5-Br-Cyt, or fluoxetine when compared to saline-treated animals (data not shown).

Behavioral effects of centrally administered 5-Br-Cyt

To determine whether the absence of effect of 5-Br-Cyt in these tests of antidepressant action was due to a lack of efficacy or a lack of penetration across the blood brain barrier, 5-Br-Cyt was infused into the ventricle. Centrally-administered 5-Br-Cyt induced a significant antidepressant-like effect in the tail suspension test (Fig. 7; $F(1,14) = 6.14$, $p = 0.026$) suggesting

that the lack of effect following peripheral administration was due to low penetration into the brain. It should be noted that difference in baseline is observed between controls of the i.p. and i.c.v. 5-Br-Cyt experiments were probably due to the effects of canula implantation, more intensive handling of animals with central drug administration and single housing of canulated mice.

DISCUSSION

We investigated the electrophysiological and behavioral properties of two cytisine derivatives, developed with the goal of identifying α4β2 nAChR-selective partial agonists with lower affinity for other nAChR subtypes including α3β4. Two compounds were selected that displayed selectivity as partial agonists of α4β2 nAChRs: 3-pyr-Cyt (a pyridine-3-yl derivative and 5-Br-Cyt (5-bromocytisine). Based on the observation that nicotinic antagonists are antidepressant-like (Mineur et al., 2007, Rabenstein et al., 2006) and that acute administration of a nicotinic agonist is not consistently antidepressant-like (Fig. 3D and 4D), along with the observation that β2KO mice have similar phenotypes to wild-type mice treated with mecamylamine or classical antidepressants (Rabenstein et al., 2006), we propose that inactivation/inhibition of α4/ β2* nAChRs is a plausible mechanism underlying the effects of cytisine derivatives in these behavioral models.

Compared to cytisine and nicotine, efficacious agonists at α3β4 and α7 receptors (Picciotto et al., 1995; Papke et al., 1994; Papke and Porter Papke, 2002, Papke et al., 2007), 3-pyr-Cyt showed little effect at α3β4 or α7 nAChRs; however, 3-pyr-Cyt was a high affinity, low efficacy partial agonist of α4β2 nAChRS (<10% activity compared to ACh). Both cytisine and 3-pyr-Cyt had differential effects on high and low sensitivity α4β2 nAChRs; however, whereas

cytisine was a more efficacious partial agonist at LS nAChRs (10 % of ACh) and a low efficacy partial agonist at HS nAChRs (less than 5 %) as has been shown previously (Bermudez and Moroni, 2006), 3-pyr-Cyt was a low efficacy partial agonist at both HS and LS nAChRs (8% and 3% of ACh, respectively). Whether the small differences in efficacy at these receptors have measurable functional consequences *in vivo* remains unclear. Taken together, these data indicate that 3-pyr-Cyt is a very weak partial nicotinic agonist at α4β2 nAChRs with much less activity at α3β4 or α7 nAChRs than cytisine. In contrast, 5-Br-Cyt did not show differential effects at LS and HS nAChRs and had an average efficacy of ~18 %, at least double that of 3-pyr-Cyt, at both receptor subtypes. 5-Br-Cyt also showed greater efficacy at α7 and α3β4 nAChRs than 3-pyr-Cyt.

Both cytisine and 3-pyr-Cyt showed antidepressant-like effects in mice across three different models of antidepressant efficacy. The cytisine used in this study was synthesized in house rather than obtained commercially as in previous studies (Mineur et al., 2007c) and was potentially more purified and therefore active at somewhat lower concentrations than in the previous study.

In the tail suspension test, 3-pyr-Cyt induced an antidepressant-like effect at lower concentrations than cytisine while acute nicotine treatment had no significant effects at any of the doses tested. This provides further evidence that blockade rather than activation of β2* nAChRs is responsible for the antidepressant-like effects of nicotinic agents (Caldarone et al., 2004; Rabenstein et al., 2006; Mineur et al., 2007c) since 3-pyr-Cyt had very low efficacy but high potency at α4β2 nAChRs and low potency at both α3β4 and α7 nAChRs. 3-pyr-Cyt may be more effective in tests of antidepressant action than cytisine because it is a low efficacy partial agonist at both LS and HS α4β2 nAChRs, whereas cytisine is a more effective agonist at LS

nAChRs. We hypothesize that *in vivo,* 3-pyr-Cyt competes with endogenous ACh, limiting α4β2*-dependent cholinergic neurotransmission, similar to a competitive antagonist. The inverted U-shaped behavioral response to cytisine and 3-pyr-Cyt in the forced swim test is most likely due to non-specific deleterious effects because higher doses of nicotinic agents tend to be aversive and hypothermic, two consequences that would increase immobility. Thus, a limited reduction of (α4β2) nAChR activity appears to be necessary to result in antidepressant-like effects without affecting other critical pathways. Additionally, a fine balance between agonism and antagonism of nAChRs may be required for efficacy in these behavioral tests (Picciotto et al., 2008). α7 nAChRs may also play a role in the response to antidepressants under specific conditions in C57BL/6J mice, since the effects of the nicotinic antagonist mecamylamine are abolished in α7 knockout mice (Rabenstein et al., 2006); however, efficacy of the nicotinic compounds tested here does not appear to depend on their intrinsic activity at α7 nAChRs. It is also possible that 3-pyr-Cyt has increased bio-availability in the brain compared to cytisine. Indeed, cytisine has poor blood brain barrier penetration, limiting its efficacy in the central nervous system (Coe et al., 2005). Similarly, the lack of behavioral effects observed with 5-Br-Cyt may be due to greatly reduced brain penetration.

In support of this hypothesis, 5-Br-Cyt, which had a greater affinity than 3-pyr-Cyt for nAChR subtypes in binding assays, and was a high affinity, low efficacy partial agonist at α4β2 nAChRs unexpectedly had no effect in the different paradigms used to assess antidepressant-like efficacy when administered peripherally. However, local infusion directly into the ventricle induced antidepressant-like effects in the tail suspension test. Thus, the most likely explanation for the lack of behavioral effect of systemic 5-Br-Cyt is that this compound is likely to have low brain penetration. One possibility could be that peripherally administered 5-Br-Cyt forms an

epoxide (at the double bond in 3-4 position of the pyridone) resulting in a hydroxyl group or a glutathione conjugate, both of which are very hydrophilic and might have altered effects at nAChRs. The lack of effect on feeding and body weight of 5-Br-Cyt suggests that the lack of behavioral effects resulting from peripheral administration is not confined to tests of antidepressant action. Additionally, we are confident these results do not reflect a failed study with 5-Br-cyt because we repeated the tests on three independent groups of animals and never saw a response.

Both cytisine and 3-pyr-Cyt were effective in the forced swim and tail suspension tests, although both compounds were less potent in the tail suspension test. While the tail suspension and forced swim tests are both tests of antidepressant efficacy that are based on a similar principle (immobility in response to a stressor), the two tests are dependent on somewhat different behavioral variables. For example, differential sensitivity to temperature could alter the results of the forced swim test but would likely have little effect on the tail suspension test. Nicotinic agents and cholinergic modulation are known to affect body temperature, likely through effects on the autonomic system such as vasoconstriction (Tritto et al., 2004). In addition, the tail suspension test is shorter in duration than the forced swim test, which may provide more time for the experimental compounds to alter behavior in the swim test, thus increasing its sensitivity. Despite the dose difference between the tests, 3-pyr-Cyt was more efficient than cytisine across paradigms. It is not known whether these compounds are fully metabolized in 24 hours and thus, there could be some carry over from one test to another, potentially explaining a stronger effect in the forced swim test compared to the tail suspension. However, given the very short half-life of cytisine, these compounds might be rapidly metabolized.

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The novelty-suppressed feeding test is only sensitive to chronic, and not acute, treatment with classical antidepressants (Dulawa and Hen, 2005) and it is thus thought to be sensitive to the neuronal adaptations that result in antidepressant effects in human depressed patients, since antidepressants must be administered for several weeks in patients to be effective. Chronic treatment with either 3-pyr-Cyt or cytisine resulted in a reduction in the time to first feed in the novelty-suppressed feeding test. This effect of 3-pyr-Cyt was only observed at one of the doses used, and the size of the effect was relatively small. Contrary to cytisine that showed an antidepressant-like effect in this test at concentration similar to those observed in both the tail suspension and the forced swim tests, 3-pyr-Cyt was only effective at a lower dose than those active in the two other tests. Many nicotinic agents can decrease feeding which can be an important confound in this paradigm. For instance, both nicotine (Jo et al., 2002) and cytisine (Mineur et al., 2007c) can decrease feeding and body weight. In the current studies, 3-pyr-Cyt modulated neither feeding nor body weight (not shown). Anxiety-like behavior could also alter performance in this test, and cytisine appears to be somewhat anxiogenic (Mineur et al., 2007c), potentially blunting the antidepressant like-effects measured in this paradigm. Interestingly, β4 null-mutant mice show reduced anxiety-like behavior in several paradigms (Salas et al., 2003) and activation of β4* nAChRs may be responsible for the ability of cytisine to increase anxiety-like behaviors. 3-pyr-Cyt should not activate α3β4 nAChRs to the same extent as cytisine, thus if the anxiogenic effects of nicotinic agents involve this nAChR subtype, 3-pyr-Cyt may not increase anxiety-like behavior to the same extent as cytisine.

In summary, we have evaluated the electrophysiological and antidepressant-like effects of two new cytisine derivatives that are weak nicotinic partial agonists with greater selectivity and affinity for β2* nAChRs. One compound, 3-pyr-Cyt, showed greater efficacy than cytisine

in three tests of antidepressant efficacy. Taken together, these data suggest that fine tuning the pharmacology of nicotinic partial agonist may result in novel therapeutic compounds to treat mood disorders.

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REFERENCES

Bermudez I and Moroni M (2006) Phosphorylation and function of alpha4beta2 receptor. J Mol Neurosci 30:97-98.

Caldarone BJ, Harrist A, Cleary MA, Beech RD, King SL and Picciotto MR (2004) High-affinity nicotinic acetylcholine receptors are required for antidepressant effects of amitriptyline on behavior and hippocampal cell proliferation. Biol Psychiatry 56:657-664.

Caldarone BJ, Karthigeyan K, Harrist A, Hunsberger JG, Wittmack E, King SL, Jatlow P and Picciotto MR (2003) Sex differences in response to oral amitriptyline in three animal models of depression in C57BL/6J mice. Psychopharmacology (Berl) 170:94-101.

Coe JW, Vetelino MG, Bashore CG, Wirtz MC, Brooks PR, Arnold EP, Lebel LA, Fox CB, Sands SB, Davis TI, Schulz DW, Rollema H, Tingley FD, 3rd and O'Neill BT (2005) In pursuit of alpha4beta2 nicotinic receptor partial agonists for smoking cessation: carbon analogs of (-)-cytisine. Bioorg Med Chem Lett 15:2974-2979.

Crawley JN (2008) Behavioral phenotyping strategies for mutant mice. Neuron 57:809-818.

Djuric VJ, Dunn E, Overstreet DH, Dragomir A and Steiner M (1999) Antidepressant effect of ingested nicotine in female rats of Flinders resistant and sensitive lines. Physiol Behav 67:533-537.

Dulawa SC and Hen R (2005) Recent advances in animal models of chronic antidepressant effects: the novelty-induced hypophagia test. Neurosci Biobehav Rev 29:771-783.

Etter JF (2006) Cytisine for smoking cessation: a literature review and a meta-analysis. Arch Intern Med 166:1553-1559.

Fitch RW, Kaneko Y, Klaperski P, Daly JW, Seitz G and Gundisch D (2005) Halogenated and isosteric cytisine derivatives with increased affinity and functional activity at nicotinic acetylcholine receptors. Bioorg Med Chem Lett 15:1221-1224.

Garcia-Colunga J, Awad JN, Miledi R (1997) Blockage of muscle and neuronal nicotinic acetylcholine receptors by fluoxetine (Prozac). Proc Natl Acad Sci U S A 94:2041-4.

George TP, Sacco KA, Vessicchio JC, Weinberger AH and Shytle RD (2008) Nicotinic Antagonist Augmentation of Selective Serotonin Reuptake Inhibitor-Refractory Major Depressive Disorder: A Preliminary Study. J Clin Psychopharmacol 28:340-344.

Glassman AH, Helzer JE, Covey LS, Cottler LB, Stetner F, Tipp JE and Johnson J (1990) Smoking, smoking cessation, and major depression. Jama 264:1546-1549.

Gohlke H, Gundisch D, Schwarz S, Seitz G, Tilotta MC and Wegge T (2002) Synthesis and nicotinic binding studies on enantiopure diazine analogues of the novel (2-chloro-5-pyridyl)-9-azabicyclo[4.2.1]non-2-ene UB-165. J Med Chem 45:1064-1072.

Gotti C, Zoli M and Clementi F (2006) Brain nicotinic acetylcholine receptors: native subtypes and their relevance. Trends Pharmacol Sci 27:482-491.

Gundisch D, London ED, Terry P, Hill GR and Mukhin AG (1999) High affinity binding of [3H]epibatidine to rat brain membranes. Neuroreport 10:1631-1636.

Imming P, Klaperski P, Stubbs MT, Seitz G and Gundisch D (2001) Syntheses and evaluation of halogenated cytisine derivatives and of bioisosteric thiocytisine as potent and selective nAChR ligands. Eur J Med Chem 36:375-388.

Janowsky DS, el-Yousef MK, Davis JM and Sekerke HJ (1972) A cholinergic-adrenergic hypothesis of mania and depression. Lancet 2:632-635.

Jo YH, Talmage DA and Role LW (2002) Nicotinic receptor-mediated effects on appetite and food intake. J Neurobiol 53:618-632.

Mineur YS, Belzung C and Crusio WE (2006) Effects of unpredictable chronic mild stress on anxiety and depression-like behavior in mice. Behav Brain Res 175:43-50.

Mineur YS, Belzung C and Crusio WE (2007a) Functional implications of decreases in neurogenesis following chronic mild stress in mice. Neuroscience 150:251-259.

Mineur YS, Picciotto MR and Sanacora G (2007b) Antidepressant-like effects of ceftriaxone in male C57BL/6J mice. Biol Psychiatry 61:250-252.

Mineur YS, Somenzi O and Picciotto MR (2007c) Cytisine, a partial agonist of high-affinity nicotinic acetylcholine receptors, has antidepressant-like properties in male C57BL/6J mice. Neuropharmacology 52:1256-1262.

Mineur YS, Abizaid A, Rao Y., Salas R, De Biasi M, Horvath TL, Gao X-B and Picciotto MR (2007) Anorexigenic proopiomelanocortin neurons in the hypothalamus are activated by nicotinic agents that decrease food intake, in Society for Neuroscience, San Diego, CA.

Mukhin AG, Gundisch D, Horti AG, Koren AO, Tamagnan G, Kimes AS, Chambers J, Vaupel DB, King SL, Picciotto MR, Innis RB and London ED (2000) 5-Iodo-A-85380, an alpha4beta2 subtype-selective ligand for nicotinic acetylcholine receptors. Mol Pharmacol 57:642-649.

Papke RL, Dwoskin LP and Crooks PA (2007) The pharmacological activity of nicotine and nornicotine on nAChRs subtypes: relevance to nicotine dependence and drug discovery. J Neurochem 101:160-167.

Papke RL and Heinemann SF (1994) Partial agonist properties of cytisine on neuronal nicotinic receptors containing the beta 2 subunit. Mol Pharmacol 45:142-149.

Papke RL and Porter Papke JK (2002) Comparative pharmacology of rat and human alpha7 nAChR conducted with net charge analysis. Br J Pharmacol 137:49-61.

Picciotto MR, Addy NA, Mineur YS and Brunzell DH (2008) It is not "either/or": Activation and desensitization of nicotinic acetylcholine receptors both contribute to behaviors related to nicotine addiction and mood. Prog Neurobiol 84:329-342.

Picciotto MR, Brunzell DH and Caldarone BJ (2002) Effect of nicotine and nicotinic receptors on anxiety and depression. Neuroreport 13:1097-1106.

Picciotto MR, Zoli M, Lena C, Bessis A, Lallemand Y, Le Novere N, Vincent P, Pich EM, Brulet P and Changeux JP (1995) Abnormal avoidance learning in mice lacking functional high-affinity nicotine receptor in the brain. Nature 374:65-67.

Quick MW and Lester RA (2002) Desensitization of neuronal nicotinic receptors. J Neurobiol 53:457-478.

Rabenstein RL, Caldarone BJ and Picciotto MR (2006) The nicotinic antagonist mecamylamine has antidepressant-like effects in wild-type but not beta2- or alpha7-nicotinic acetylcholine receptor subunit knockout mice. Psychopharmacology (Berl) 189:395-401.

Reitstetter R, Lukas RJ and Gruener R (1999) Dependence of nicotinic acetylcholine receptor recovery from desensitization on the duration of agonist exposure. J Pharmacol Exp Ther 289:656-660.

Salas R, Pieri F, Fung B, Dani JA and De Biasi M (2003) Altered anxiety-related responses in mutant mice lacking the beta4 subunit of the nicotinic receptor. J Neurosci 23:6255-6263.

Salin-Pascual RJ, de la Fuente JR, Galicia-Polo L and Drucker-Colin R (1995) Effects of transderman nicotine on mood and sleep in nonsmoking major depressed patients. Psychopharmacology (Berl) 121:476-479.

Semba J, Mataki C, Yamada S, Nankai M and Toru M (1998) Antidepressant-like effects of chronic nicotine on learned helplessness paradigm in rats. Biol Psychiatry 43:389-391.

Tizabi Y, Overstreet DH, Rezvani AH, Louis VA, Clark E, Jr., Janowsky DS and Kling MA (1999) Antidepressant effects of nicotine in an animal model of depression. Psychopharmacology (Berl) 142:193-199.

Tritto T, McCallum SE, Waddle SA, Hutton SR, Paylor R, Collins AC and Marks MJ (2004) Null mutant analysis of responses to nicotine: deletion of beta2 nicotinic acetylcholine receptor subunit but not alpha7 subunit reduces sensitivity to nicotine-induced locomotor depression and hypothermia. Nicotine Tob Res 6:145-158.

Zhou Y, Nelson ME, Kuryatov A, Choi C, Cooper J and Lindstrom J (2003) Human alpha4beta2 acetylcholine receptors formed from linked subunits. J Neurosci 23:9004-9015.

Zoli M, Lena C, Picciotto MR and Changeux JP (1998) Identification of four classes of brain nicotinic receptors using beta2 mutant mice. J Neurosci 18:4461-4472.

FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1 – Partial agonist properties of cytisine and derivatives at high and low sensitivity α4β4 nAChRs. Structure and activity of the compounds used in this study: A, B) cytisine; C, D) 3-pyr-Cyt; E, F) 5-Br-Cyt. Ki values have been published previously and were determined by competition for [^{3}H]epibatidine and [^{3}H]MLA binding sites using radioligand binding in rat forebrain (α4/β2*, α7*), pig adrenals (α3/β4*) and *Torpedo californica* electroplax ($(\alpha1)_2\beta1\gamma\delta$) membrane fractions (Gundisch et al., 1999; Mukhin et al., 2000; Gohlke et al., 2002). The high sensitivity form of α4β2 (α4(2)β2(3)), was generated by the co-expression of RNA coding the β2–6–α4 concatamer along with monomeric β2 (Zhou et al., 2003). The low sensitivity form of α4β2 (α4(3)β2(2)) was generated by the co-expression of RNA coding the β2–6–α4 concatamer along with monomeric α4 (Zhou et al., 2003). The data plotted (B, D, F) represent the average responses (±SEM) from at least four oocytes at each concentration and have been normalized relative to the maximum ACh-evoked responses for each receptor subtype (see methods).

Figure 2 - Effects of of 5-Br-Cyt, and 3-pyr-Cyt on α4β2, α3β4 and α7 nAChR subtypes. A) 5-Br-Cyt and B) 3-pyr-Cyt were tested for their ability to activate α3β4 and α7 type nAChRs expressed in *Xenopus* oocytes and compared to activity at α4β2 nAChRs (Papke and Heinemann, 1994; Papke and Porter Papke, 2002; Papke et al., 2007). The data plotted represent the average responses (±SEM) from at least four oocytes at each concentration and have been normalized relative to the ACh maximum responses for each receptor subtype (see methods). C) The effects of 3-pyr-Cyt on α4β2 receptors were also studied in co-application experiments with ACh. Oocytes were tested for their responses to applications of 30 μM ACh and these

responses were compared to the responses evoked by 30 μM ACh co-applied with increasing concentrations of 3-pyr-Cyt. The co-application responses of at least 4 oocytes (±SEM) are plotted, normalized to the responses of the same oocytes to ACh alone. Note that in these experiments (A-C) the α4β2 receptors were formed from the co-expression of α4 and β2 monomers and so represent a mixed population of the high and low sensitivity subtypes.

Figure 3 – Effects of cytisine, 3-pyr-Cyt and 5-Br-Cyt, nicotine and fluoxetine in the tail suspension test. Total time spent immobile in the tail suspension test by C57BL/6J male mice treated with various doses of A) cytisine, B) 3-pyr-Cyt C) 5-Br-Cyt, D) nicotine and fluoxetine. n = 10 per treatment group. X values indicate the dose injected in mg/kg. Error bars represent SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4– Effects of cytisine, 3-pyr-Cyt and 5-Br-Cyt in the forced swim test. Total time spent immobile in the forced swim test by C57BL/6J male mice treated with various doses of A) cytisine, B) 3-pyr-Cyt,C) 5-Br-Cyt, D) nicotine and fluoxetine n = 10 per treatment group. X values indicate the dose injected in mg/kg. Error bars represent SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 5– Effects of acute cytisine, nicotine, 3-pyr-Cyt and 5-Br-Cyt on locomotor activity. Horizontal activity was measured as total number of beam breaks in C57BL/6J male mice 20 min after cytisine, 3-pyr-Cyt, 5-Br-Cyt or nicotine. n = 10 per treatment group. Error bars represent SEM.

Figure 6 – Effects of cytisine, 3-pyr-Cyt and 5-Br-Cyt in the novelty-suppressed feeding test. Time required for C57BL/6J male mice to initiate a feeding episode in the novelty suppressed feeding test after chronic injection (15 days) of various doses of A) cytisine, B) 3-pyr-Cyt and C) 5-Br-Cyt. n = 10 per treatment group. X-axis values indicate the dose injected in mg/kg. Error bars represent SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 7 – Effects of centrally-administered 5-Br-Cyt in the tail suspension test. Total time spent immobile in the tail suspension test by C57BL/6J male mice after 50 ng i.c.v. of 5-Br-Cyt. n=8 per treatment group. Error bars represent SEM. * $p < 0.05$.

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TABLE 1

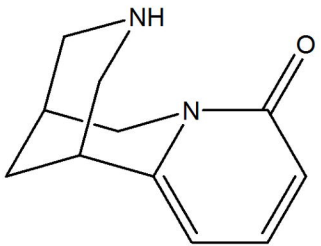
		Activation		
Agonist	**Subunits**	**I_{max}**	**n**	**EC_{50}**
ACh	hα4(3)β2(2)	1	0.76 ± 0.1	73 ± 12
ACh	hα4(2)β2(3)	1	0.9 ± 0.1	1.7 ± 0.3
5-Br-Cyt	hα4(3)β2(2)	0.17 ± 0.01	1.2 ± 0.1	15.1 ± 1.7
5-Br-Cyt	hα4(2)β2(3)	0.17 ± 0.01	1.0 ± 0.1	13.3 ± 1.7
5-Br-Cyt	mixed α4β2	0.162 ± 0.005	1.4 ± 0.1	38 ± 3
5-Br-Cyt	hα3β4	0.66 ± 0.04	1.66 ± 0.16	124 ± 13
5-Br-Cyt	hα7	0.386 ± 0.015	2.4 ± 0.4	18.1 ± 1.8
3-pyr-Cyt	hα4(3)β2(2)	0.03 ± 0.001	0.81 ± 0.03	31 ± 3
3-pyr-Cyt	hα4(2)β2(3)	0.08 ± 0.01	1.0 ± 0.2	12 ± 4
3-pyr-Cyt	mixed α4β2	0.023 ± 0.002	1.2 ± 0.2	33 ± 6
Cytisine	hα4(3)β2(2)	0.10 ± 0.02	0.35 ± 0.06	12.7 ± 8.7
Cytisine	hα4(2)β2(3)	≤ 0.05	N. A.	N.A.
		Inhibition of 30 μM Ach-evoked response		
Drug	**Subunits**	**n**	**IC_{50}**	
3-pyr-Cyt	mixed α4β2	-0.8 ± 0.1	60 ± 12	

Maximal responses and potencies of the compounds used in this study with respect to activation of nAChRs expressed in oocytes or inhibition of the response to 30 μM ACh. The parameters are those of the fits to the Hill equation, (see methods). I_{max} values are expressed relative to ACh I_{max}. Note that the responses of α4(2)β2(3) receptors were too small to obtain reliable curve fits (N. A.).

Figure 1

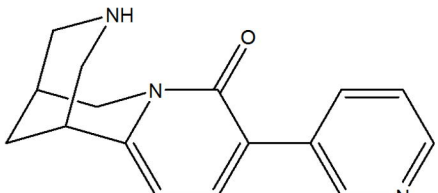
A

Cytisine



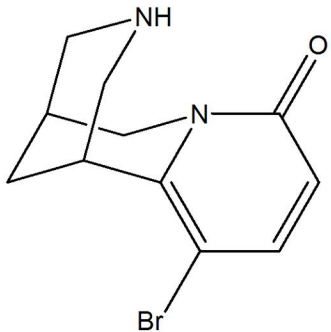
C

3-pyr-Cyt



E

5-Br-Cyt



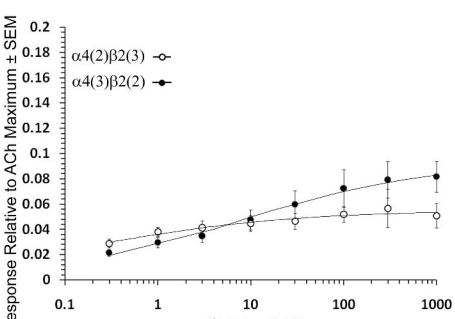
Ki's

α4β2*: 0.120 nM
α3β4*: 19 nM
α7*: 250 nM

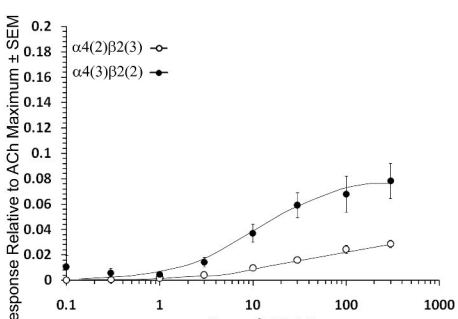
α4β2*: 0.91 nM
α3β4*: 119 nM
α7*: 1100 nM

α4β2*: 0.308 nM
α3β4*: 3.8 nM
α7*: 28 nM

B



D



F

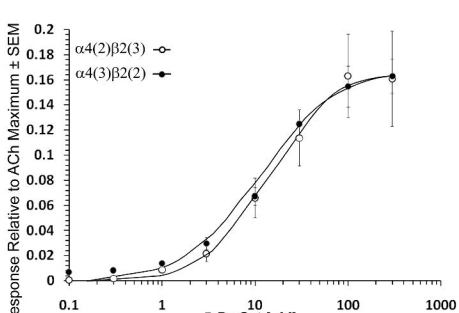


Figure 2

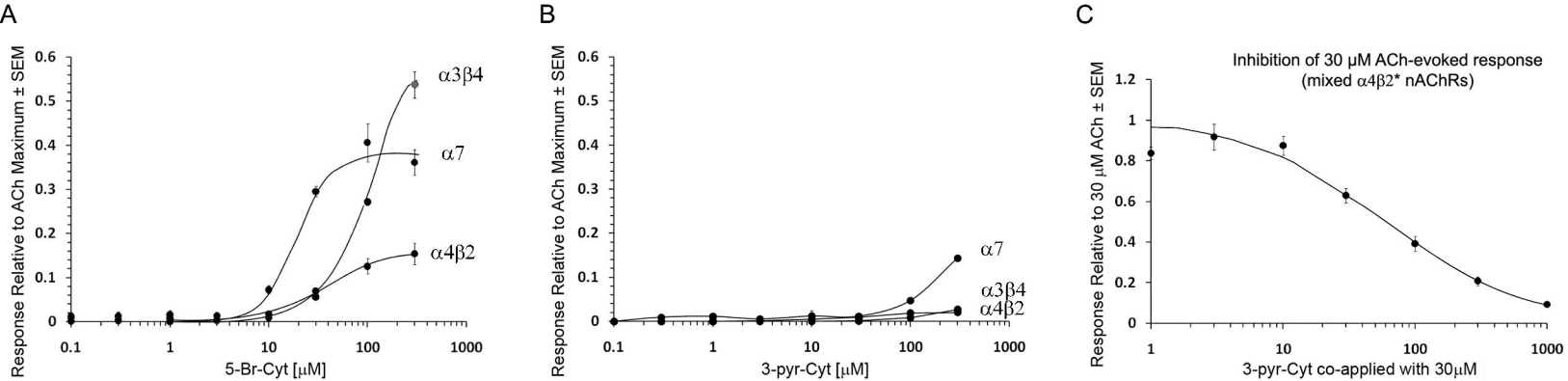

A
Response Relative to ACh Maximum ± SEM
0.6
0.5
0.4
0.3
0.2
0.1
0
0.1
1
10
100
1000
5-Br-Cyt [μM]
α3β4
α7
α4β2
B
Response Relative to ACh Maximum ± SEM
0.6
0.5
0.4
0.3
0.2
0.1
0
0.1
1
10
100
1000
3-pyr-Cyt [μM]
α7
α3β4
α4β2
C
Inhibition of 30 μM ACh-evoked response
(mixed α4β2* nAChRs)
Response Relative to 30 μM ACh ± SEM
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
1000
3-pyr-Cyt co-applied with 30μM

Figure 3

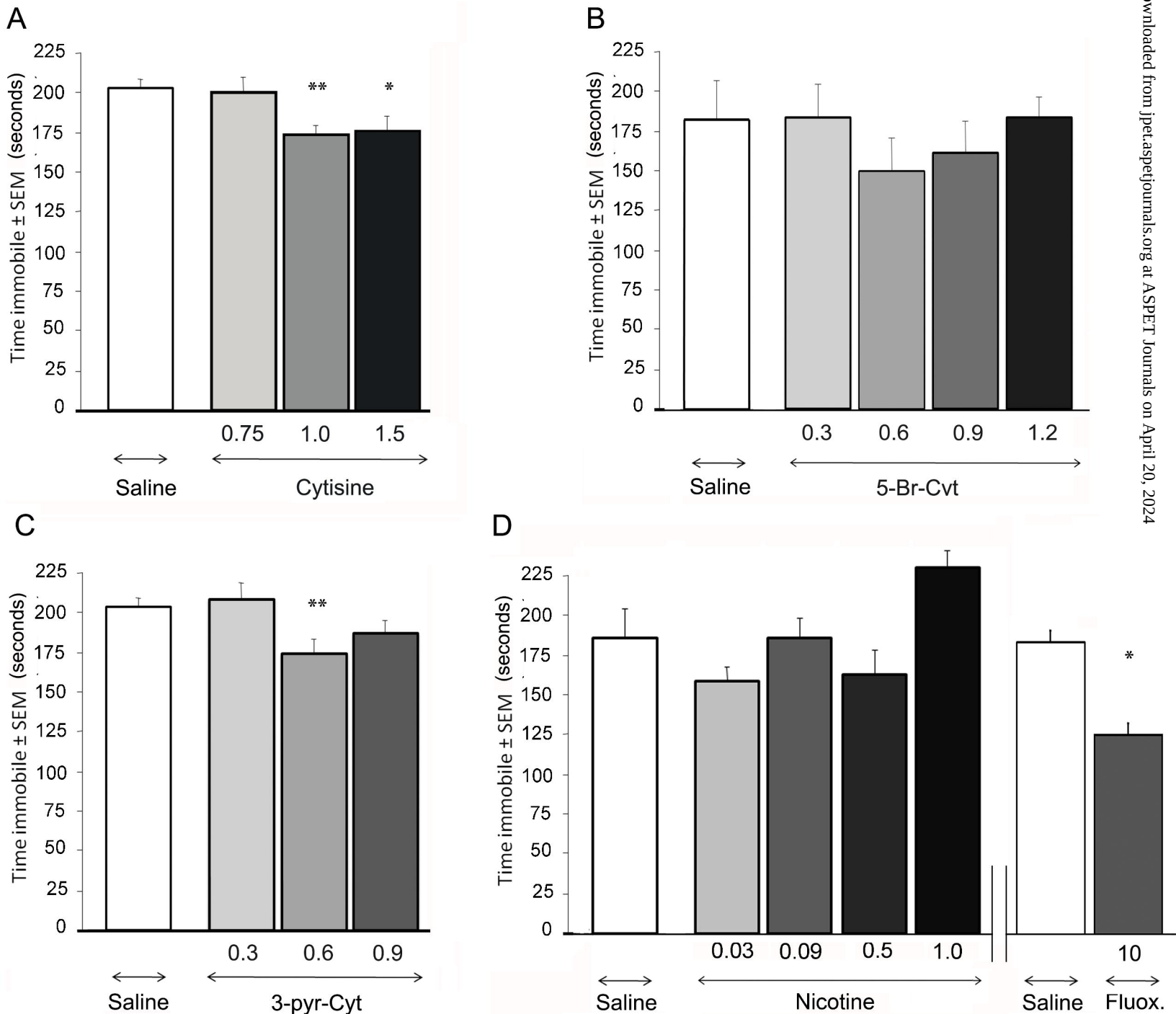


Figure 4

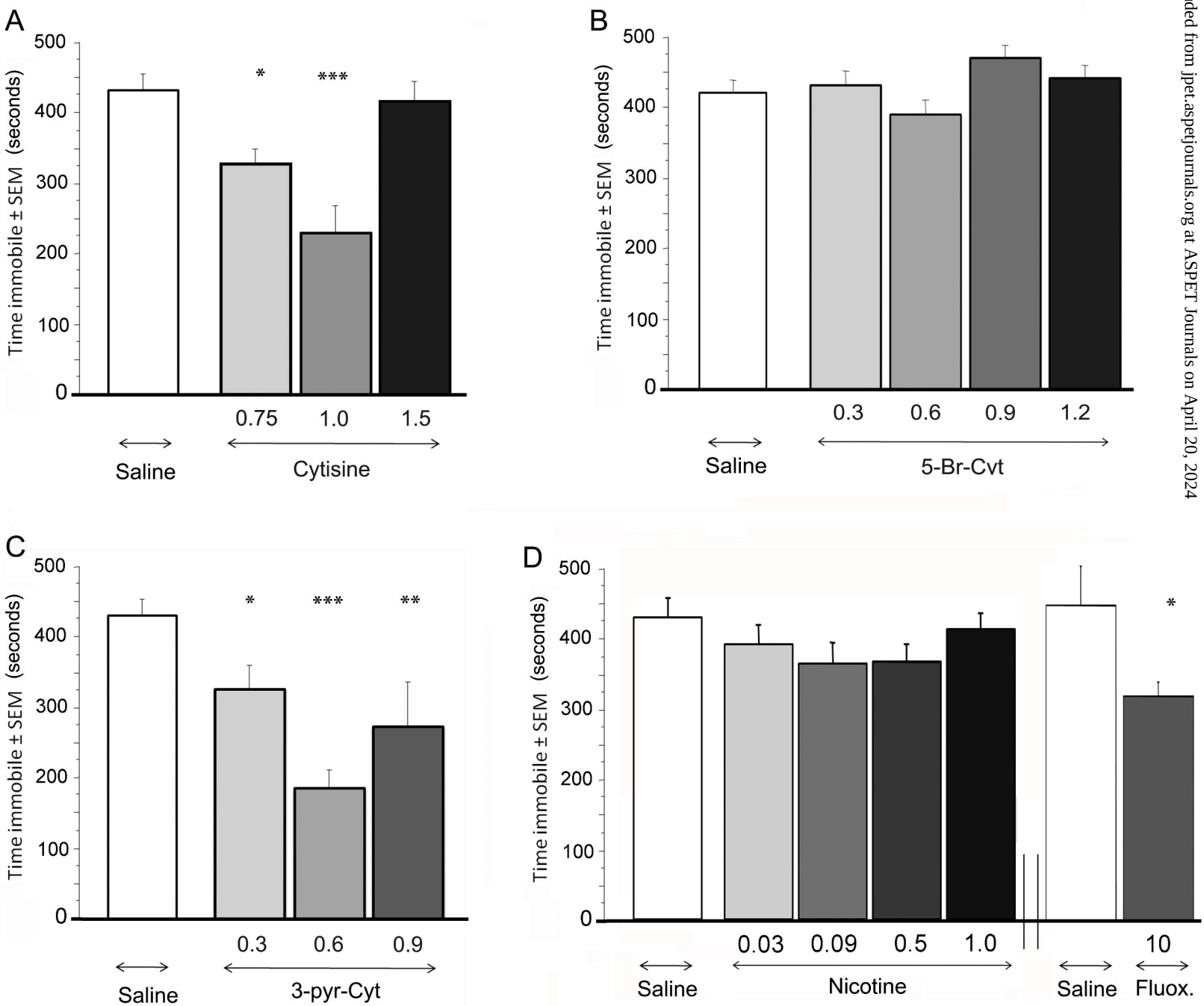


Figure 5

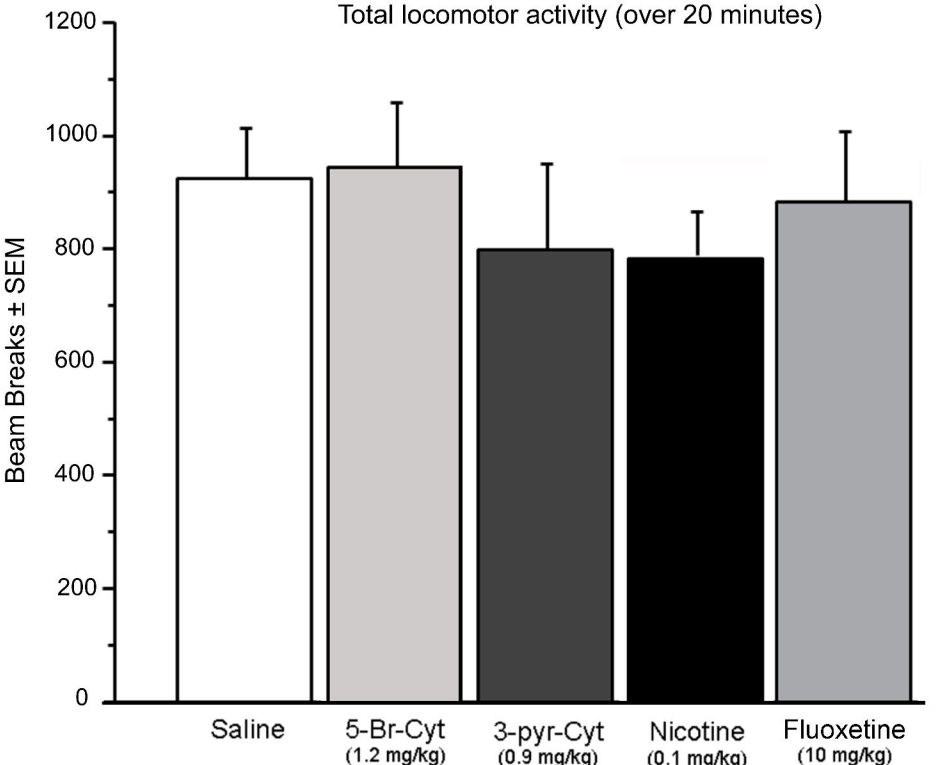

Total locomotor activity (over 20 minutes)
Beam Breaks ± SEM
1200
1000
800
600
400
200
0
Saline
5-Br-Cyt
(1.2 mg/kg)
3-pyr-Cyt
(0.9 mg/kg)
Nicotine
(0.1 mg/kg)
Fluoxetine
(10 mg/kg)

Figure 6

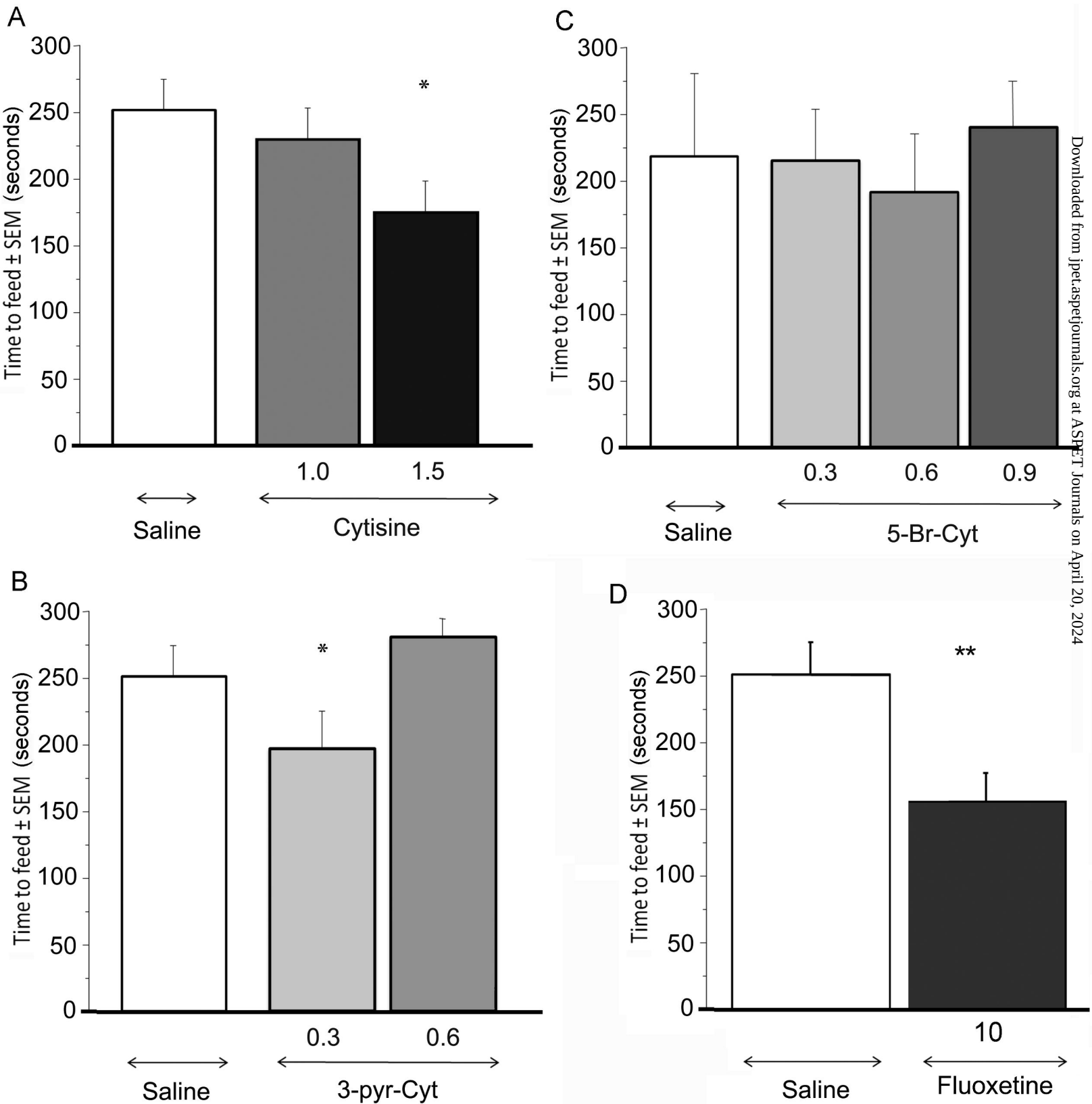


Figure 7